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# Electrophoretic Protein Analysis of Red blood cell Membrane Proteins upon Ionic and Non-ionic detergent lysis

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**Abstract:**
Red blood cells (RBC) are anucleated atypical cellular architecture with a plasma membrane envelope where the glycoproteins are oriented along the lipid bilayer. RBC proteins are classified as peripheral and integral proteins based on its location along plasma membrane. The present study explored the extraction efficiency of various detergents such as Triton X100, Sodium dodecyl sulphate (SDS), Cetypyridinium chloride (CPC), alone and its combinations along with protease inhibitor cocktail for RBC membrane proteins analyzed in an elctrophoretogram. The study showed that RBC protein extraction was much better in combination of Triton X100 and SDS as evident by increased number of protein bands in coomassie brilliant blue R stained SDS gel, compared to CPC combined Triton X100 lysis buffer. The study hence showed that RBC proteins can be extracted with combination of non-ionic detergent and anionic detergent.
**Key words:** Red blood cells, Triton X100, Sodium dodecyl sulphate, Cetyl pyridinium chloride

## INTRODUCTION

Red blood cells (RBC) is unique among mammalian cells in structure which have no nucleus and cytoplasmic organelles. Primary function of RBC is the transport of oxygen ($O_2$) and carbon dioxide ($CO_2$) between the tissue and lungs. There is large number of proteins found in RBC. Currently more than 50 membrane proteins are recognized [1, 2]. Based on orientation in plasma membrane there are two groups of proteins; they are Integral and Peripheral proteins.

Integral proteins or intrinsic protein are permanently attached to main membrane, it penetrate through the lipid bilayer. All transmembrane proteins are integral proteins. Examples are Glycophorins, Band 3 and Aquaporin 1. It contains hydrophobic part that interacts with membrane phospholipid [2]. Extractions of integral proteins need the use of strong agents that solubilise the phospholipids bilayer to release proteins. Peripheral proteins or extrinsic proteins are temporarily attached to lipid layer or they bind to other integral proteins. It includes actin, spectrin, and tropomyosin, ankyrin, protein 4.1 and protein 4.2. Spectrin is a large, cytoskeletal protein composed of α and β subunits .mainly involved in cell adhesion and cell

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spreading. Mutation in Spectrin leads to cancer, anaemia, and ataxia [3]. Peripheral proteins are separated by disrupting the hydrogen bond between proteins in the surface of phospholipids bilayer, without destroying the membrane. Cytoskeletal proteins are visible below the lipid layer and helps in maintaining shape, providing mechanical support to plasma membrane. The present study is focused on RBC protein extraction efficiency of different detergents based on its ionic properties.

Membrane proteins can be separated by using detergents which can be mild like Triton X100 or strong detergents like Sodium dodecyl sulphate (SDS). Detergents have both hydrophilic and hydrophobic properties which help to separate membrane proteins leading to disruption of cell membrane. Surfactant concentration plays an important role in the solubilization of membrane [4]. At low concentration, some changes can occur in the membrane permeability whereas at higher concentration there might be drastic effects like cell lysis [5, 6]. Now, there are many detergent developed to improve solubilization of proteins. Three types of detergents are used in the present study base on the charge.

Ionic detergents bear either positive (cationic) or negative (anionic) charges in their polar head group. Anionic detergents have negative charged sulphate group as hydrophilic head. Cationic detergents contain positive charged ammonium group. Sodium dodecyl sulphate (SDS) is an anionic detergent. It totally disrupts membranes and degrades proteins by breaking protein-protein interaction. Polar group of SDS bind to proteins and cause structural change [7]. Cetylpyridiniumchloride (CPC) is a cationic detergent also is a quaternary ammonium compound and a monocationic surfactant. It has the ability to remove membrane lipid and promote cell lysis [7]. Non ionic detergents contain molecules with head group uncharged. TritonX100 is a non ionic surfactant used to solubilise membrane proteins. It is a milder detergent. Triton X100 commonly used in biomembrane studies, derived from polyoxyethylene and contained an alkylphenyl hydrophobic group. It has bulky non polar heads that don't penetrate into water soluble proteins and do not have cooperative property. Some proteins and lipids are resistant to Triton X100 called detergent resistant membranes DRMs [8, 9].

A common method for analysis of the integral and peripheral proteins from

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ghost membrane (an erythrocyte after loss of its haemoglobin) of RBC is SDS Polyacrylamide gel electrophoresis (SDS-PAGE). It is an analytical technique to separate protein based on molecular weight.

## Materials and methods

Triton X100, Coomassie Brilliant blue-R (HiMedia), Sodium dodecyl sulphate (Affymetrix-USB), Cetyl Pyridinium chloride (CPC), Protease inhibitory cocktail (Sigma), Electrophoretic apparatus (Bio Rad). All other chemicals used for SDS-PAGE were purchased from HiMedia, SRL and Sigma.

## Methodology

RBC ghost membrane preparations were done according to standard protocol with slight modifications [10]. Approximately 1mL of whole blood in EDTA coated tube was added with 4 mL of diluents composed of 120 mM NaCl, 10 mM EDTA, 5 mM sodium citrate and 5 mM Tris-HCl, pH 7.4. and vortexed. The homogenate was centrifuiged at 3500 rpm for 10 minutes. The pellet obtained was added with 4 mL of ice cold 5 mM Tris-HCl and 1 mM EDTA followed by vortexing. This was followed by centrifugation at 3500 rpm for 10 minutes. The pellet was resuspended in 5 mM Tris-HCl, pH 7.4, 500 mM NaCl and 1 mM EDTA and centrifuged at 16,000g for 10 minutes. The faint pink colored membrane free of haemoglobin obtained as RBC ghost was suspended in 5 mM Tris-HCl and lysed with lysis buffer composed of SDS, Triton X100 and CPC or in their various combinations supplemented with Protease inhibitor cocktail. The protein content in RBC ghost lysate was quantitated by Bicinchoninic acid (BCA) method. Approximately 200 µg protein per well along with 2X Laemlli buffer was used for loading on to SDS-PAGE.

## RESULTS

In the present study we have analyzed the electrophoretic pattern of erythrocyte proteins upon treatment with different detergents having ionic and non ionic nature. The anionic detergent was Sodium dodecylsulphate (SDS), cationic detergent was Cetylpyridiniumchloride (CPC) and non ionic detergent was Triton X100. The extraction efficiency of various detergents and its combination for erythrocyte proteins was compared with the help of Sodium dodecylsulphate –Polyacrylamide gel electrophoresis (SDS-PAGE). Bands was stained with Coomassie brilliant blue R 250 followed by destaining using combination of acetic acid ,methanol, distilled water.

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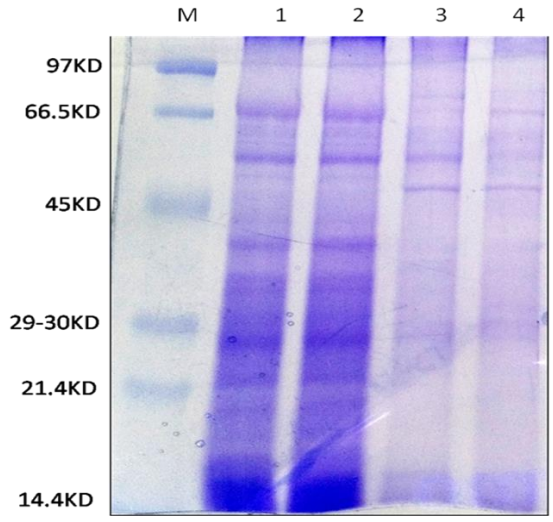


Figure -1

**Figure 1 . Electrophoretic erythrocyte protein profiling in presence of ionic (SDS) and nonionic(Triton X 100)detergents.**

**M – Pre stained Marker, LANE 1 - 0.5% SDS +2% Triton X 100, LANE 2 - 0.25%SDS +1% Triton X 100,LANE 3 – 2% Triton X 100, LANE 4 -1% Triton X 100**

In figure1, we have analysed the electrophoretic pattern of erythrocyte proteins upon lysis with non-ionic detergent TritonX100 and its combination with ionic detergent SDS (Sodiumdodecylsulphate).This pattern reveals that extraction efficiency of erythrocyte proteins were higher in combination of SDS and Triton X100 than Triton X100 alone treated samples.

The first topmost prominent band appeared was in the region of 66KD as evident from marker position. From previous studies with regards to erythrocyte proteins, the band could be Tropomyosin having molecular weight 66 KD in lane 1 (0.5%SDS + 2%Triton) and lane2 (0.25%SDS +1%Triton). These bands corresponding to 66KD were faiding in lane 3 (2% Triton X100) and lane 4 (1% TritonX100). The second most prominent band which appeared in the range between 45-65KD could be either Dematin (52KD) or Actin (50KD) in lane 1 and 2 (combination of SDS andTritonX100).These bands appeared faint in lane 3 (2% triton) and lane 4 (1% triton).Extraction efficiency was lower in Triton X100 compared to combination of SDS and Triton X100. In 3$^{rd}$and4$^{th}$ lane, TritonX100 alone treated sample showed a prominent band between the ranges of 45-55KD could be flotllin, which was absent in combination (0.5% SDS + 2% TritonX100) treatments. There was a marginal appearance of a protein band in the region closer to 72-75KD could be protein 4.1, which was specifically appeared in 2% TritonX100 alone treated samples (lane 3). A prominent protein band below the region of 42 KD based on marker position in SDS +TritonX100 (lane 1, 2) sample could be the protein band6 or glyceraldehydes 3 phosphate dehydrogenase. This was absent or faint in TritonX100 alone treated samples (lane 3, 4).

In the region between 30-35KD, there appeared two distinct bands in combination of SDS and TritonX100 (lane1, 2) could be either Aquaporin 1or stomatin .These bands were absent in 2% and 1%Triton X100 alone treated samples (lane3, 4). In the region of 29KD, there appeared a band which could be stomatin (29-33KD), which showed better intensity in combination treatment (lane 1, 2), which were relatively faint in Triton alone treated sample (lane 3, 4). There was a band which appeared in combination of SDS and Triton (lane1, 2) in the region between 20-25 KD could be peroxiredoxin which was absent in TritonX100 alone treated samples.

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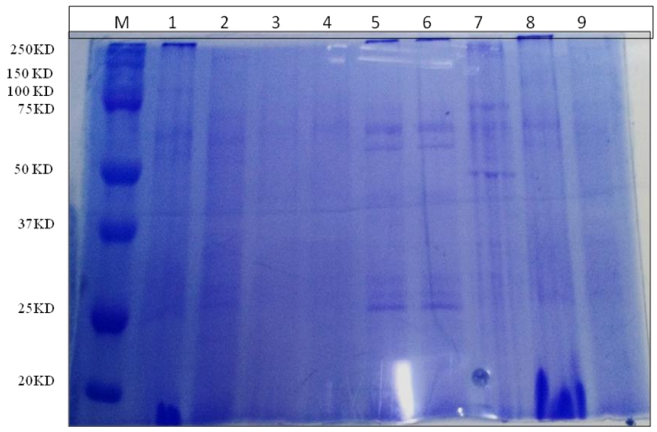


**Figure – 2**

**Figure 2. Erythrocyte membrane protein extraction upon ionic (SDS) non ionic (Triton X100) and cationic (CPC) detergents lysis**

**M – Precision plus protein marker, LANE 1 – 0.5 % SDS, LANE 2 – 1 %SDS, LANE 3 – 0.5 %CPC + 2 %TritonX100, LANE 4 – 0.5% CPC + 2% TritonX100, LANE 5 – 1% SDS +1% TritonX100, LANE 6 - 1% SDS +2% TritonX100, LANE 7 – 2% TritonX100, LANE 8 – 0.5% SDS+1% CPC, LANE 9 – 2 % SDS**

In figure 2, we have analysed the protein profiling of erythrocyte membrane using different detergent lysis with various combinations. This pattern revealed that extraction efficiency was comparatively better in combination 1 % SDS and 1 to 2 % Triton X100. The prominent band were appeared in combination of SDS and Triton X100 (lane5, 6) could be tropomyosin (66KDprotein). The second prominent band can be seen in SDS+TritonX100 combination (lane5, 6) could be dematin (52KD) or actin (50 KD) which were absent in other treatments. Still, we found two extra prominent band in 2% TritonX100 alone treated sample (lane7) might be protein4.1 (75 KD) and flotllin (47KD) but these two were absent or faint in combination of Triton X100 with SDS (lane 5, 6). Some prominent bands were appeared in the range between 25KD-37KD could be Aquaporin 1(32 KD) or stomatin (29-33KD) in lane 5 and 6 (SDS+TritonX100), which was absent in other treatments. Combination of CPC with Triton X100 (lane3, 4) and 1%SDS (lane 8) showed low extraction efficiency compared with others. Hence, the band patterns in electrophoretogram in figure 1 and 2 showed that over all the extraction efficiency of RBC proteins was higher in combination of SDS and Triton X100.

The results of electrophoretic analysis of erythrocyte proteins upon detergents lysis with SDS, CPC and Triton X100 individually or in various combinations were analyzed in Figure 1 and 2. From the two elctrophoretograms in figure 1 and 2 we observed that extraction efficiency was much better for Triton X 100 and SDS combinations compared to Triton X and SDS per se mediated lysis buffer. This was evident by more number of protein bands visible in Triton X 100 and SDS combination lysis buffer. Further, we have also found that Cetylpyridinium chloride (CPC), the cationic detergent was not a good extraction buffer in combination with Triton X 100 and alone revealed by the band intensity pattern in elctrophoretogram in figure 2.

## DISCUSSION

Erythrocytes are anucleated cells and easy to be characterized by electrophoresis as reported from early

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studies (11, 12). RBC has membrane proteins associated with maintaining integrity of cells. Distribution of lipids, detergent concentration, electrostatic and hydrophobic interaction of lipids and protein affect the interaction between membrane protein and detergents based on hexagonal lattice study in RBC cytoskeleton (4, 13). Prominent difference in protein profiling with different detergents were found with the help of SDS-PAGE. Anionic detergent, SDS is a solubiliser molecule which was used to extract erythrocyte proteins (14). SDS bound to both to their hydrophobic and hydrophilic part, so it often denatures membrane protein. Strong anionic detergent caused cell lysis within seconds. But extraction efficiency was quite less in SDS alone treated sample observed in the present study could be attributed to increased micelle volume in RBC plasma membrane. From our study we have found that ionic detergents are good solubilisers *per se* but not suitable for efficient protein extraction. TritonX100 is a milder non ionic detergent which caused slower cell lysis. It contained non polar head which were so easy to penetrate cell membrane but do not penetrate into water soluble protein. With the help of non ionic detergent, a few more protein could be obtained in SDS-PAGE such as tropomyosin, stomatin, aquaporin and Glyceraldehyde 3 dehydrogenase. But some specific proteins can be seen only in Triton X100 treatment, which is absent in other treatments such as Band 4.1 and flotilin. During solubilisation, Triton X 100 replaced lipid molecule in contact with the hydrophobic domain of integral membrane proteins and leading to the extraction of mixture of RBC proteins (15, 16). Integral membrane proteins such as lipid raft depended integral protein stomatin (33KD), Aquaporin 1(32KD) also appeared in Triton X100 treated sample, and even intensified upon combination with SDS. Some membrane proteins are resistant to Triton X100 called Detergent resistant membrane (DRMs). Lipid raft contain high amount of cholesterol and sphingolipid. DRMs include flotillin1, 2, stomatin (9). Flotililin and stomatin were identified as lipid raft proteins which tend to be altered in diseases with altered RBC morphology such as hereditary stomatocytosis (17). In our study, we found that flotllin appeared only in 2% triton X100 lysis and stomatin in combination of 0.25-0.5% SDS with Triton X 100. There was yet another protein Band 6 also known as glyceraldehydes 3 phosphate dehydrogenase which is an enzyme appeared distinctively in erythrocyte protein extraction using combination of 0.25-0.5% SDS and to 1-2% Triton X100 (figure 1) . The protein which

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could be extracted out of TritonX100 and SDS combination at various concentrations includes tropomyosin, actin, dematin and stomatin. Flotllin, and protein 4.1, were absent in other combinations such as cationic detergent CPC with either Triton X 100 or with SDS. The cationic detergent we used for the study was Cetylpyridiniumchloride (CPC). Its mechanism of action is not understood. When CPC treated alone samples, there were quite a few erythrocyte proteins appeared in the gel. The identified protein appeared within the region between 72-75KD could be protein4.1. The above mentioned band was even absent in combination of CPC and TritonX 100. So we can conclude that CPC alone treated and combination with Triton X100 showed low extraction efficiency. The individual bands compared with different detergent treatments, marked differences can be seen. The same protein are stained stronger in mixed extraction and weaker in alone treatments. Effective detergents for solubilization of erythrocytes can be a useful tool in analysing various RBC proteins and its alterations in diseases pertaining to haematological disorders. So this study throws light on using combination of SDS and Triton X 100 as a detergent cocktail to study RBC proteins.

## CONCLUSION

In this study, we focused on erythrocyte protein profiling using different detergents. The SDS-PAGE method with the help of different detergents could make a powerful tool for the extraction of membrane proteins. We have extraction protocols by different detergent composition of solubilisation buffer included charged anionic (Sodiumdodecylsulphate), uncharged non-ionic (Triton X 100) and cationic (Cetylpyridiniumchloride) detergents and their combinations. The analysis of protein profiling by SDS PAGE showed that combination of SDS (anionic) and TritonX100 (non ionic) showed better protein extraction efficiency. Relevance of study is that, this technique is used further for RBC membrane studies and its characterisations and biochemical features.

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